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Determination of Hyperin in the Fruits of *Acanthopanax* Species by High Performance Liquid Chromatography

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Abstract – The content of hyperin in *Acanthopanax* species was determined by high performance liquid chromatography (HPLC). Hyperin was quantified by a reverse-phase column with elution program [initially gradient solvent (acetonitrile : water = 85 : 15 to 80 : 20 for 20 min), then isocratic solvent (acetonitrile : water = 80 : 20 for 20 min), and finally gradient solvent (acetonitrile : water = 80 : 20 to 65 : 35 for 20 min)]. UV detection was conducted at 210 nm. The content of hyperin in the fruits of *Acanthopanax* was measured in the species *A. chiisanensis* (2.04 mg/g), *A. sessiliflorus* (1.13 mg/g), *A. divaricatus* (0.98 mg/g), *A. koreanum* (0.75 mg/g) and *A. senticosus* (0.05 mg/g). The content of hyperin in *A. chiisanensis* was higher than that of other *Acanthopanax* species.
Keywords – *Acanthopanax*, Araliaceae, quantitative analysis, flavonoid, hyperin

Introduction

Acanthopanax species, belonging to the family Araliaceae, are perennial herbaceous species distributed in Korea, China, and Japan. They have traditionally been used as a tonic and a sedative, as well as in the treatment of rheumatism and diabetes (Perry and Metzger, 1980; Yook, 1990).

Many studies have shown that *Acanthopanax* exhibits a variety of pharmacological activities such as anti-bacterial, anti-cancer, anti-inflammatory, anti-gout, anti-hepatitis, anti-hyperglycemic, anti-leishmanicidic, anti-oxidant, anti-pyretic, choleretic, hemostatic anti-xanthine oxidase, immunostimulatory, hypo-cholesterolemic, and radio-protectant (Davydov and Krikorian, 2000). These plants have been widely used as health supplements in Korea. Triterpenoid saponins such as sliphioside F, copteroside B, hederagenin 3-*O*-β-D-glucuronopyranoside 6'-*O*-methyl ester, and gypsogenin 3-*O*-β-D-glucuronide from *A. senticosus* fruits are reported to be pancreatic lipase inhibitors (Fang *et al.*, 2007). Aqueous extracts from the fruits of *A. senticosus* are reported to have antioxidant and antimicrobial effects (Kim *et al.*, 2006). Isolation and characterization of several lignans, coumarins, flavonoids, and terpenes from *Acanthopanax* species have previously been reported (Shin and Lee, 2002). Among them, hyperin, quantified by HPLC from the stem of *A. senticosus* and *A. sessiliflorus* (Lee *et al.*, 2004a), has been reported to show antioxidant (Han *et al.*, 2000), anti-inflammatory (Lee *et al.*, 2004b) and aldose reductase inhibitory activities (Lee *et al.*, 2003).

However, there has been no report on the determination of hyperin in the fruits of *Acanthopanax* species. Therefore, the determination of hyperin in fruits of *Acanthopanax* species was conducted by efficient and simple analytical methods.

Experimental

Plant materials – *Acanthopanax* species (*Acanthopanax chiisanensis*, *A. divaricatus*, *A. koreanum*, *A. senticosus,* and *A. sessiliflorus*) were cultivated and collected by Wolga Agro-Products Co. Ltd., Gongju, Korea, and botanically identified by Prof. S. H. Cho, Gongju National University of Education, Korea.

Instruments and reagents – ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AVANCE 400 NMR spectrometer (Rheinstetten, Germany) in DMSO using TMS as an internal standard. The mass spectrometry (MS) was measured with a Jeol JMS-AX505WA mass spectrometer (Tokyo, Japan) with a direct inlet. HPLC chromatograms were recorded with a GILSON 305 system pump with a GILSON 188 system UV/VIS detector (Villiers le Bel, France). Water and

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acetonitrile used in this research were of HPLC grade, and all other reagents were of analytical grade.

Preparation of hyperin – Air-dried grounded powder of *A. chiisanensis* fruits (1.5 kg) was extracted with MeOH under reflux for 3 h. The MeOH extract (524.5 g) was suspended in water, and then fractionated successively with equal volumes of *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH (yield: 28.5, 23.7, 19.9, and 82.5 g, respectively). Among them, a portion of the EtOAc fraction (5.0 g) was chromatographed on a silica gel, and eluted with a stepwise gradient of $CHCl_3$-MeOH (100% $CHCl_3$ up to 100% MeOH). Hyperin was isolated from the 15% MeOH fraction after recrystallization with MeOH.

Hyperin: EI-MS *m/z* 302 (100) $[M\text{-Gal}]^+$, 273 (7.3), 245 (4.2), 207 (11.1), 153 (6.1), 137 (7.4), 128 (7.1); FAB-MS *m/z* 465 $[M+H]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H 12.64 (1H, s, 5-OH), 7.67 (1H, dd, J = 2.0, 8.5 Hz, H-6'), 7.53 (1H, d, J = 2.0 Hz, H-2'), 6.82 (1H, d, J = 8.5 Hz, H-5'), 6.41 (1H, d, J = 1.9 Hz, H-8), 6.21 (1H, d, J = 1.9 Hz, H-6), 5.38 (1H, d, J = 7.8 Hz, galactosyl H-1"); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C 177.9 (C=O), 164.5 (C-7), 161.6 (C-5), 156.6 (C-2, C-9), 148.9 (C-4'), 145.2 (C-3'), 133.9 (C-3), 122.4 (C-6'), 121.5 (C-1'), 116.3 (C-5'), 115.6 (C-2'), 104.3 (C-10), 102.2 (C-1"), 99.1 (C-6), 93.9 (C-8), 76.2 (C-5"), 73.6 (C-3"), 71.6 (C-2"), 68.3 (C-4"), 60.5 (C-6").

Sample preparation – For the analysis of hyperin in *Acanthopanax* species, 300 mg of each of the fruits from *Acanthopanax* species (*A. chiisanensis*, *A. divaricatus*, *A. koreanum*, *A. senticosus,* and *A. sessiliflorus*) were extracted with 50% MeOH (20 ml × 3 times) by reflux and evaporated in vacuo. The residue was dissolved in 6 ml of 50% MeOH and filtered with a 0.45 μm filter. The resulting solution was used for HPLC analysis.

HPLC condition – The HPLC separation of hyperin for qualitative and quantitative analysis was performed using a reverse phase system. A Nucleodur 100-5 C18 ec (4.6 × 250 mm, 5 μm) column was used, with a mobile phase consisting of acetonitrile and water. The elution program was initially gradient solvent (acetonitrile : water = 85 : 15 to 80 : 20 for 20 min), then isocratic solvent (acetonitrile : water = 80 : 20 for 20 min), and finally gradient solvent (acetonitrile : water = 80 : 20 to 65 : 35 for 20 min). UV detection was conducted at 210 nm. The injection volume was 10 μl and the flow rate was 1 ml/min. All injections were performed in triplicate.

Calibration – A stock solution (3 mg/6 ml) of hyperin isolated from *A. chiisanensis* was prepared in 50% MeOH, and blended with 50% MeOH, repeatedly, diminishing the solution content to 50% percent to

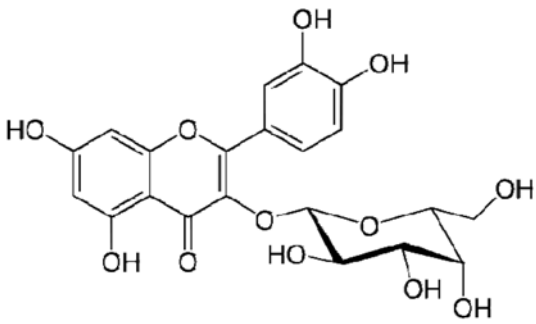

Fig. 1. Structure of hyperin.

different concentrations. The contents of the analytics were determined from the corresponding calibration curves. The calibration functions of hyperin are calculated with peak area (Y), concentration (X, μg/10 μl), and mean values (n = 5) ± standard deviation.

Results and Discussion

A chromatographic separation of the MeOH extract from the fruits of *A. chiisanensis* led to the isolation of hyperin (Fig. 1). The presence of hyperin in the fruits of *A. chiisanensis* was identified by spectroscopy. The typical flavonoid signals were identified in the ^{1}H-NMR spectrum. The singlet at δ 12.64 showed the typical aromatic 5-OH of the flavonoid A ring. The proton signals at δ 7.67 (dd, J = 2.0, 8.5 Hz), 7.53 (d, J = 2.0 Hz) and 6.82 (d, J = 8.5 Hz) showed the ABX splitting type of the B ring. The proton signals at δ 3.00~5.00 represented glycoside. The ^{13}C-NMR spectrum showed C=O at δ 177.9. The signals at δ 102.2 represented an anomeric carbon of sugar moiety. The FAB-MS showed an $[M+H]^+$ ion at *m/z* 465. The molecular formula was determined to be $C_{21}H_{20}O_{12}$. Accordingly, the structure was elucidated as hyperin by comparing its spectral data in the literature (Lee *et al.*, 2002).

Hyperin was detected in *Acanthopanax senticosus* (Chen *et al.*, 2002), *Epimedium koreanum* (Sha *et al.*, 1995), *Hypericum perforatum* (Wu *et al.*, 2002), *Sanguisorba officinalis* (Sha *et al.*, 1998) and apple (Lommen *et al.*, 2000; Chinnici *et al.*, 2004). Hyperin contents of 0.47 and 0.14 mg/g were identified in the stems of *A. senticosus* and *A. sessiliflorus*, respectively. No hyperin was detected in the root of *A. senticosus,* but 0.30 mg/g in that of *A. sessiliflorus* (Lee *et al.*, 2004a). However, there have been no reports on the determination of hyperin in the fruits of *Acanthopanax* species.

Hyperin content in the various *Acanthopanax* fruits was determined by HPLC. The standard curve for hyperin is

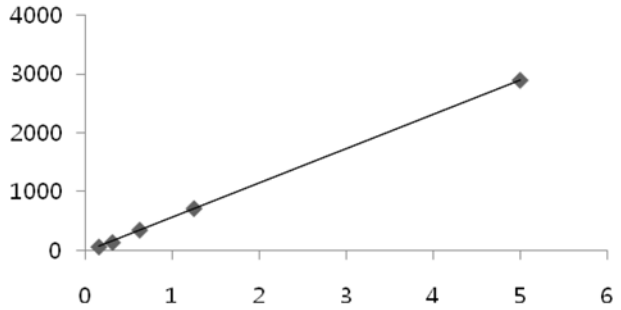

Fig. 2. Calibration curve for hyperin (X axis, μg/10 μl; Y axis, Area).

Table 1. Content of hyperin in the fruits of *Acanthopanax* species

Samples	Content (mg/g)
Acanthopanax chiisanensis	2.04 ± 0.25
A. sessiliflorus	1.13 ± 0.02
A. divaricatus	0.98 ± 0.09
A. koreanum	0.75 ± 0.12
A. senticosus	0.05 ± 0.01

Data are given as the mean ± S.D. (n = 3) in $mg \cdot g^{-1}$ dried samples.

$Y = 583.82X - 18.686$ ($r^2 = 0.9999$) (Fig. 2). Hyperin was shown at the retention time 21.73 min. The retention time of the expected peak of hyperin in *A. chiisanensis* was the same as that of the standard compound. Table 1 shows that hyperin was detected in the fruits of *A. chiisanensis* (2.04 mg/g), *A. sessiliflorus* (1.13 mg/g), *A. divaricatus* (0.98 mg/g), *A. koreanum* (0.75 mg/g), and *A. senticosus* (0.05 mg/g).

In previous papers (Kang *et al.*, 2003; Lee *et al.*, 2007), *Acanthopanax* species were be classified into two groups, with low concentration of chiisanoside (*A. senticosus*, and *A. koreanum*) and high concentration (*A. chiisanensis, A. divaricatus,* and *A. sessiliflorus*). The content of hyperin was similar to that of chiisanoside. In particular, the content of hyperin in *A. chiisanensis* was 40 times than that of *A. senticosus*.

It is very important that hyperin, the main anti-inflammatory agent in *A. chiisanensis,* has been identified in the fruits of other *Acanthopanax* species. The presence of hyperin in the fruits of *Acanthopanax* species is especially important in agricultural crop production for increasing the amounts of clinically available medicine and health supplements. Accordingly, these results demonstrate that *Acanthopanax* species containing hyperin have promising potential as new additives to natural products for the development of fruit juice, wine, food products, and health supplements in Korea.

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